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Epidemiological Research Report

Section 2. Number 16

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Experimental Research on Toxic Fractions of U.S.W. Cholera Vaccine

~~(Secret)~~

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General

Scholars at home and abroad claim that the true nature of bacterial toxins can be found in proteins, particularly nucleoproteins, and in carbohydrates. Differences and variations in the chemical properties of bacteria can be understood when it is realized that their chemical composition as well as their toxic properties vary with the type of bacteria. The conspicuous advances made recently on the study of bacterial carbohydrates are commanding attention from other fields.

The study of cholera toxins also has advanced, as is evidenced by numerous publications on the subject. Successive research on the virulence of carbohydrates in cholera bacteria are being performed. Recently KUROYA published his report on toxic protein fractions.

A solution of the chemical properties of cholera toxins will furnish not only vitally important basic data but will provide a guiding policy essential to vaccine manufacture and preventive medicine.

The specific mission of this laboratory is to study supersonic wave-treated cholera vaccines. Studies on cholera toxins have been extremely rare. If supersonic wave-treated vaccines are to be improved in quality over the old cholera vaccines it would be inadequate to emphasize merely the research concerned with the chemical properties of their toxins. Full consideration must be given toward providing ideal conditions under which such studies can be conducted.

It has been reported already by this laboratory that the U.S.W. cholera vaccine has proved to be effective and has produced excellent results when examined for difficult immunity reactions. Based on this, the first step in studying the toxins of the supersonic wave-treated cholera vaccine was the extraction of fractions by applying the relatively simple precipitation reaction according to a fixed procedure. An examination of their toxic distribution revealed extremely interesting signs. A part of the results will be presented in this report.

Chapter I. Test materials

A. Bacterial strain: The type used in the experiment was the Kitani strain possessing a virulence of 0.3 mg.

B. Vaccine manufacture: Suspensions were prepared by adding the bacterial culture (developed after incubating for 18 hours at 37°C in an agar medium) to a physiological saline solution at the rate of 10 mg per cc. After subjecting the bacterial suspension to the action of supersonic waves (600,000 cycles per second) for 15 minutes, peptone and slant cultures were made to determine whether or not the bacterial cells had been destroyed. Those indicating cellular destruction were used in the experiment.

Chapter II. Experimental procedure

A. Preliminary tests: In order to avoid the powerful precipitant reaction the maximum precipitation range at a fixed hydrogen ion concentration was determined by adding acetic acid or trichloroacetic acid. Before starting the main tests the preliminary

tests outlined below were completed.

1. Precipitation range with acetic acid:

Reagents of PH 2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0 and 8.0 were prepared with a Japan Pharmacopeia acetic acid solution. Two cc of each reagent was injected into a sterile test tube stoppered with cotton. An equal volume (2.0 cc) of the bacterial suspension was added and the test tubes were shaken thoroughly. After being allowed to stand for over 18 hours in a refrigerator or at room temperature the precipitation conditions were examined.

Table 1. Precipitation results with acetic acid of varying PH values.

(Degree of precipitation indicated by + ++ +++ ++++; negative precipitation by (-).)

PH	2.0	3.0	3.5	4.0	4.5	5.0	6.0	7.0	8.0
Room temperature (29.5°C)	(+) No change	+++ Heavy precipitation at bottom; top clear	++++ Extremely heavy precipitation at bottom; top clear	++ Heavy precipitation at bottom; top clear	+ Light precipitation at bottom; top turbid	(-) Uniform turbidity	(-) Uniform turbidity	(-) Uniform turbidity	(-) Uniform turbidity
Refrigerator (4°C)	(-) No change	+ Light precipitation at bottom; top turbid	+++ Heavy precipitation at bottom; top not completely clear	++ Precipitation at bottom; top not completely clear	+ Small amount of precipitation at bottom; top somewhat turbid	(-) Uniform turbidity	(-) Uniform turbidity	(-) Uniform turbidity	(-) Uniform turbidity

The results in Table 1 show a strong acid agglutination-precipitation at PH 3.0 - 4.0 and a clear supernatant fluid. Uniform turbidity was displayed at values above PH 4.0. The preliminary test was repeated in order to detect possible errors in these results. The proper precipitation point was established at PH 3.5.

2. Precipitation range with trichloroacetic acid:

The trichloroacetic acid test followed the same procedure as that in the acetic acid test. The results appeared to indicate complete agglutination - precipitation in the PH 3.0 - 3.6 range at room temperature but a positive precipitation point such as that resulting from the acetic acid test could not be obtained.

B. Main tests: The main tests were commenced after learning through preliminary tests that complete agglutination - precipitation could be produced with a PH 3.5 acetic acid solution.

1. Acetic acid precipitation treatment: A 300-cc capacity separating funnel was filled with 150 cc of bacterial solution and 150 cc of acetic acid solution. The mixture was allowed to stand after being thoroughly shaken for several minutes. A cloud-like turbidity and partial precipitation resulted several hours later. The greater portion precipitated after 24 hours; suspended particles causing turbidity gradually precipitated. This was processed in a Sakuma type centrifuge on the third day. Approximately 10 cc of the content was measured into each vessel and centrifuged at 4000 r.p.m. for 20 minutes. A slightly gray precipitate (1) and a transparent supernatant fluid were obtained.

Acetic acid precipitate (A): Ten times by volume of alcohol was added to the centrifuged precipitate (1) which was obtained through acetic acid precipitation described above. This was allowed to stand in a refrigerator for three days, after which the alcohol was replaced. A small volume of ether was added to the sediments produced by centrifuging, and suction filtration was performed. A black powdery substance was obtained upon drying the sediments. Yield was 0.128 g.

2. Acetic acid soluble fractions

Acetone precipitate (B): The supernatant fluid (II), measuring approximately 530 cc, which remained after the acetic acid precipitate was removed by centrifugal separation, was placed in an evaporating dish and evaporated with the aid of an electric fan. Precipitation was effected with acetone after the fluid had been condensed to a volume of about 56 cc. This was allowed to stand in a refrigerator for a 24-hour period. The supernatant acetone was removed by decantation. A suitable volume of acetone was added immediately to the sediments. This was stirred and the supernatant fluid and sediments (grey powder) were separated by suction filtration.

The sediments were washed first with acetone and then with ether and dried in a vacuum dessicator over phosphoric anhydride. The substance was a grey powder; yield was 0.7876 g.

3. Hydrated acetone soluble fractions: The supernatant fluid obtained by decantation and the filtrate remaining after

suction filtration were combined and evaporated. Ether was added to the minute yield of sediments. This was stirred and extraction was allowed to continue. Two fractions, the ether extract and the ether insoluble sediment, were obtained after filtration.

Ether extract (C): A small quantity of a black, amorphous sediment was produced when ether was added to the ether soluble portion and evaporated with an electric fan. Yield was merely 0.0036 g.

Ether insoluble sediment (D): The ether insoluble sediment was dried into a powder. A yellowish-grey precipitate was obtained; yield was 0.0046 g.

Chapter III. Virulence tests on animals.

A. German mice weighing 10 - 13 g each were selected for the virulence tests. Five mice comprised a group.

B. Test materials consisted of 0.02 g each of the different powders dissolved in 25 cc of physiological saline solution.

C. Injections of the test solutions were made intraperitoneally in 0.1 cc, 0.2 cc, 0.3 cc, 0.4 cc, 0.5 cc, 0.6 cc, 0.7 cc, 0.8 cc, 0.9 cc and 1.0 cc doses. Test results were evaluated after observing whether the mice had survived or died during the three-day period following the injections.

D. Virulence test results.

Precipitate (A) produced death with a 1.8 mg dose (M.L.D.).

Acetone precipitate (B) which was obtained by precipitating the condensed supernatant fluid with acetone indicated a virulence of 1.8 mg (M.L.D. was equal to that of the acetic acid precipitate). Unfortunately this substance (B) was not analyzed. In all probability this higher compound was part protein and contained a small amount of polysaccharides.

The ether soluble substance (C), which before evaporation remained as a supernatant fluid instead of precipitating when acetone was added, failed to produce death with a 7.0 mg dose.

The ether insoluble portion (D) from (C) did not cause death with a 7.0 mg dose.

Chapter IV. Ash and nitrogen content of fraction precipitates.

For the purpose of grasping a general picture concerning the toxicity of fraction precipitates from the U.S.W. antigen, rough data on chemical properties were obtained by measuring the ash and nitrogen contents of fractions giving the highest yields. Because of the small yield analysis was not possible on fractions other than the acetic acid precipitates.

Results of experiment

Acetic acid precipitate (A)

Nitrogen soluble substance	$\frac{N}{50}$ SO_4H_2 consumption	Nitrogen
6.73 mg	2.53 cc	10.53%
6.25 mg	2.26 cc	10.22%

The ash content of the precipitates was 0.39 percent; the nitrogen content of the organic matter was 10.38 percent. Nitrogen value was low compared to that ordinarily indicated by proteins. Studies on whether they are nucleoproteins or glycoproteins and whether they contain non-nitrogenous matter or a substance such as a polysaccharide are expected at a later date.

KUROYA reported a nitrogen value of approximately 13 percent for a toxic substance contained in a protein precipitated from an autolysis product of cholera bacteria.

Ether extract (B): The nitrogen test substance weighed 13.3 mg; $\frac{N}{50}$ SO_4H_2 consumption was not evident. Although practically the major portion of the supernatant fluid was believed to have consisted of fatty substances, details could not be learned due to the small yield. The main points of the experiment are listed in Table 2; a summary of the procedure is given in Table 3.

Table 2. Virulence tests on fractions

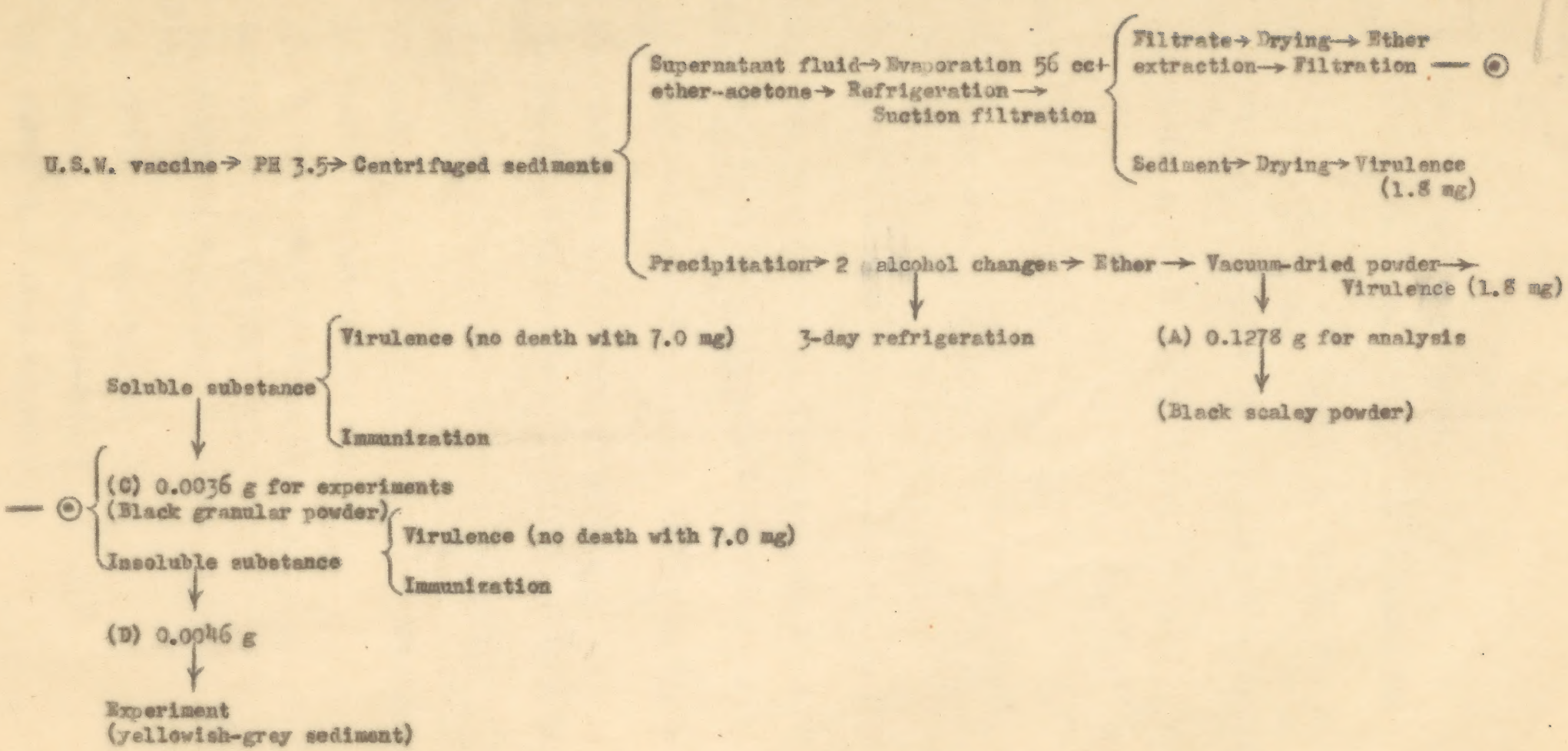
Code	Fraction	Yield	Virulence	Nitrogen	Ash
(A)	Precipitate from acetic acid precipitation treatment followed by centrifugalization.	0.1278g	1.8 mg	10.38%	0.39%
(B)	Acetone precipitate from condensed supernatant fluid in acetic acid precipitation.	0.7876g	1.8 mg	-	-
(C)	Ether soluble product, a hydrated acetone extract of condensed supernatant fluid obtained in acetic acid precipitation.	0.0036g	Failed to cause death at 7.0 mg	0	-
(D)	Ether insoluble product of above.	0.0046g	Failed to cause death at 7.0 mg	-	-

Chapter V. Observations

The above facts reveal that the acetic acid precipitate and the acetone precipitate are toxic and require 1.5 mg doses to kill mice. Since the bacterial strain from which this antigen was derived possesses an M.L.D. of 0.3 mg it is extremely interesting to note that its virulence is approximately six times that of the precipitates. An examination of the antigenic strength of these substances through immunization tests is desired. If they prove to be highly effective it will mean that a method for producing a superior antigenic substance has been discovered. Immunization tests will be performed with newly prepared antigenic materials when the opportunity for continuing such a study is presented.

The ether soluble subetance and the ether insoluble substance failed to produce death with a 7.0 mg dose. The shortage of antigenic materials prevented the testing of higher dosages. This point must be studied at a later date.

Table 3. Acetic acid fraction test on U.S.W. supersonic wave-treated vaccine



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